

Isolating Viruses from
Finished Water

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16. ABSTRACT The reduction of enteroviruses and rotaviruses was studied at a full scale 205 mgd water treatment plant involving chemical flocculation, sand filtration and chlorination. Reduction of enteroviruses and rotaviruses averaged 81% and 93%, respectively, for the complete treatment process. The greatest reduction of enteroviruses occurred during prechlorination/flocculation and filtration, while the greatest reduction of rotaviruses occurred during pre-chlorination/clarification and final chlorination. Enteroviruses or rotaviruses occurred in 24% of the finished water samples containing levels of free chlorine (>0.2 mg/l), and meeting coliform bacteria (1/100 ml) and turbidity (1 NTU) standards. Although major plant deficiencies may have been responsible for occurrence of viruses in the finished water, the results of this study indicate that finished water having measureable levels of free residual chlorine and meeting standards for coliform bacteria and turbidity cannot be assumed to be virus free.		
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Isolating Viruses From Finished Water

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Reduction of enteroviruses and rotaviruses averaged 81 and 93 percent, respectively, at a full-scale 205-mgd (776-ML/d) plant whose treatment train includes chemical flocculation, sand filtration, and chlorination. The highest reduction of enteroviruses occurred during prechlorination-flocculation and filtration, whereas the highest reduction of rotaviruses occurred during prechlorination-clarification and final chlorination. Enteroviruses or rotaviruses occurred in 24 percent of the finished water samples, which had >0.2 mg free chlorine/L and met coliform bacteria (1/100 mL) and turbidity (1 ntu) standards. Although major plant deficiencies may have been responsible for the occurrence of viruses in the finished water, the results of this study indicate that finished water, with measurable levels of free residual chlorine and meeting standards for coliform bacteria and turbidity, cannot be assumed to be virus free.

Since the turn of the century, it has been well known that there is a significant risk of contracting infectious diseases from the ingestion of sewage-contaminated water or food. The spread of enteric bacterial disease by this route has been controlled by the application of bacterial standards and modern treatment processes for drinking water and sewage.

Isolation of viruses from treated drinking water¹⁻⁵ has generated concern that water treatment methods may not always adequately ensure that water designated for human consumption is virus free. In fact, viruses have been isolated from drinking water that met recommended levels of coliform bacteria, chlorine, and turbidity.^{5,6} These findings have raised serious questions about the adequacy of bacterial standards for judging the sanitary quality of water in terms of its potential for the transmission of viral disease.

The majority of processes used to treat potable water sources are capable of reducing the numbers of viruses. Enteric viruses have, however, been shown to be less effectively removed or inactivated than indicator bacteria, and much of the available information on the removal of enteric viruses by water

treatment processes is based on laboratory studies with members of the enterovirus group (particularly, poliovirus type 1) and a few selected coliphages. However, not all enteric viruses are likely to be removed with as high a rate of efficiency by a given water treatment process. Laboratory studies have shown that poliovirus is more effectively removed from water by alum flocculation and from sewage by adsorption to sludge flocs than is rotavirus.⁷ Also, under field conditions at an activated-sludge sewage treatment plant, enteroviruses appeared to be more effectively removed than were rotaviruses.⁸ Naturally occurring viruses may, however, be more resistant to chlorination than are prototype laboratory strains.⁹

Few studies have been done on the removal of naturally occurring enteroviruses in actual drinking water treatment plants under field conditions. Results of a previous report,⁶ which describes the isolation of enteroviruses and rotaviruses from finished water in a distribution system and at a treatment plant, prompted this more extensive investigation at the same site. This study reports on the occurrence of enteric viruses within a full-scale water treatment plant following each treatment

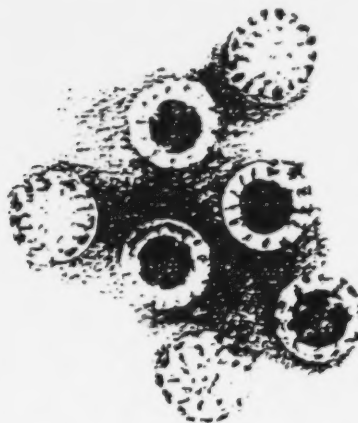
process, including clarification, filtration, and chlorination.

Materials and methods

Water treatment plant. In this study the site was a 205-mgd (776-ML/d) water treatment plant supplied with raw river water that is conveyed approximately 11 mi (17 km) by canal via two pumping stations. Untreated wastewater is discharged into a shallow lake that feeds into the river from which the source water is taken.

The treatment facility consists of five separate plants constructed over a 20-year period (1955-75). Each plant has a treatment sequence that consists of chemical addition (liquid alum, 31 mg/L; polymer, *0.6 mg/L) followed by hydraulic mixing, flocculation, clarification with pre- and postchlorination, filtration through rapid sand filters or automatic valveless sand filters, and final chlorination. Pump rates were reported at 39.63 gpm (25 L/s). Samples collected included raw or intake water, clarified water, filtered water, and finished water from three of the five plants. Four trips to the plant were made over a two-year period, and 20-40 samples were collected during each trip.

Virus sample collection and concentration procedures. Samples of 9.8 to 756 L were collected according to procedures described elsewhere¹⁰ using electropositive adsorbent filters.† An electric, 1-hp (0.7-kW) centrifugal pump was used to collect samples that could not be collected directly from taps. In-line injectors‡ were used where necessary to inject sodium thiosulfate into water to neutralize the chlorine before passage through the virus-adsorbing filters.¹¹ On each day that samples were collected, finished



water samples were collected first and intake water samples last to avoid possible contamination of the finished water samples. Separate filter housings and hoses were used for each sampling location. The filter housings, hoses, and pumps were disinfected by contact with free chlorine (>5 mg/L) for 30 min.

Viruses adsorbed onto the filter were eluted with 1-L of 3 percent beef extract, § pH 9.5. The filter housing was filled with the beef extract solution and allowed to remain for 2–5 min, then forced out of the housing with air. The pH of the eluate was immediately adjusted to 7.0–7.5 by adding 1 N HCl. Samples were packed in crushed ice (for one to five days) before shipment to the laboratory at the University of Arizona, where they were frozen at –20°C for preservation before final processing.

Primary filter eluates were thawed and reconcentrated from a volume of 1 L to approximately 36 mL by an organic flocculation method described by Katzenelson et al.¹² The final concentrate was divided into three equal volumes: 10 mL were examined for enteroviruses and 10 mL were examined for rotaviruses; the third portion was stored at –40°C for future use.

Enterovirus and rotavirus detection. All samples for enterovirus analysis were inoculated onto monolayers of BGM cells, 2 mL/75 cm² plastic flask, overlaid with maintenance medium and observed for cytopathic effects (CPE) for a period of 21 days. Samples that did not produce CPE were passed an additional two times through monolayers of BGM cells and observed for 14 days for CPE.

Those samples positive for CPE were passed a second time in the BGM cell line and then plaqued as a final confirmatory

test using an agar overlay method.¹³ Virus isolates were subsequently identified by serum neutralization tests using the Lim-Melnick enterovirus antibody serum pools.¹⁴

Rotaviruses were detected by inoculating the sample onto cultures of MA-104 cells⁵ in glass chamber slides** in the presence of a medium containing trypsin.†† After 24 h of incubation at 37°C, the medium was aspirated off, the chamber removed, and the monolayers fixed in –20°C methanol for 5 min. The slides were then stained by the immunofluorescent assay described by Smith and Gerba.⁸

Grab samples. One-litre grab samples and virus samples were taken simultaneously. Bacterial densities as well as the physical-chemical properties of the water were determined from each grab sample. A portion of each sample was transported on ice to the University of Arizona for turbidity and coliphage analysis.

Physical-chemical measurements of water quality. Total and free chlorine levels were determined by the DPD colorimetric method using a chemical chlorine kit.‡‡ The pH was determined using a digital pH meter,§§ which was standardized several times each day with pH 4, 7, and 10 buffers. The pH probe was disinfected between samples by placing it in a solution of 1 N HCl for 10 min. After being transported to the University of Arizona, samples were analyzed for turbidity.***

Bacterial assays. Bacterial analyses were performed immediately with the grab samples for standard plate count bacteria, total and fecal coliform bacteria, and fecal streptococci bacteria. The membrane filtration method (MF)

was used¹⁵ to enumerate the bacteria and employed the use of nutrient pad kits††† for consistency and ease of handling. Appropriate dilutions were made, and 10-mL, 50-mL, and 100-mL, and, for some samples, 200-mL volumes were filtered. Enterococci were also enumerated in 30 samples using the MF method developed by Levin et al.¹⁶

Positive colonies isolated on the membrane filters for total and fecal coliforms from finished drinking water were transferred to nutrient agar slants for transport. The coliform isolates were submitted to presumptive and confirmatory tests. A percentage of presumed non-coliform colonies isolated from the membrane filters were also tested in the same manner to detect false negatives.

Positive fecal coliform isolates were confirmed by placing them in EC broth††† and incubating them at 44.5°C for 48 h; gas production constituted a positive test.

For this study, selected colonies of fecal streptococci were confirmed by their growth on KF fecal streptococcus media and subsequent development of the typical colonies, ranging in color from pink to red.

Coliphage assays. Bacteriophage concentrations were determined in the grab samples as well as in each 1 MDS filter eluate, before freezing, using *Escherichia*

*Catfloc or Superfloc, Calgon, Pittsburgh, Pa.

†1 MDS Virosoorb filters, AMF CUNO, Meriden, Conn.

‡DEMA, St. Louis, Mo.

§Scott Laboratories, Fiskeville, R.I.

**Lab-Tek, Miles Labs., Naperville, Ill.

††0.5 µg/mL Sigma Type IX, Sigma, St. Louis, Mo.

‡‡CN-70, Hach Chemical Co., Loveland, Colo.

§§Model 18800, Hach Chemical Co., Loveland, Colo.

***Turbidimeter 2100A, Hach Chemical Co., Loveland, Colo.

†††Nalge, Rochester, N.Y.

‡‡‡Difco, Detroit, Mich.

TABLE 1
Summary of mean recoveries* for turbidity, bacteria, and viruses after successive drinking water treatments

Type of Water	Date of Sampling	Turbidity ntu	Total Plate Count per mL	Total Coliforms	Fecal Coliforms	Fecal Streptococci	Enterococci	Coliphage per L (Direct)	Coliphage per L (Concentrate)	Enterovirus† CPE percent	Rotavirus per 100 L
Raw	3/82	6.4	240	600	203	2 436	†	700	417	80	408
	1/83	3.4	864	70	6	15 205	1165	3300	1119	50	0
	4/83	10.9	440	264	147	2 766	†	1700	599	25	14
Grand means§		8.0	465	216	66	4 452	1165	1769	680	47	15
Clarified	3/82	5.7	126	0	0	13	†	500	4	60	20
	1/83	3.8	94	4	0	1 120	31	100	1	11	3
Grand means		4.5	103	2	1	189	31	177	2	29	6
Filtered	1/83	0.9	9	1	0	47	2	6	1	0	12
Grand means		0.9	9	1	0	47	2	6	1	0	12
Finished	3/82	1.2	20	0	0	6	†	0	0.8	44	3
	1/83	0.8	12	1	0	67	6	1	0.9	4	1
	4/83	1.1	6	3	1	8	†	1	0.9	0	1
Grand means		1.0	9	1.5	1	20	6	1	0.9	9	1

*All bacterial and viral numbers are geometric means; turbidities are arithmetic means.

†Percentage of samples positive for virus

‡Not determined

§Weighted by number of samples per trip

TABLE 2
Summary of enteric viruses isolated from all samples* after successive drinking water treatment processes

Type of Water	Number of Samples	Viruses							
		Enteroviruses		Rotaviruses		Both Enteroviruses and Rotaviruses†		Total Enteric Viruses	
		NP‡	percent	NP	percent	NP	percent	NP	percent
Raw	19	9	47	9	47	5	26	13	68
Clarified	14	4	29	5	36	2	10	7	50
Filtered	8	0	0	3	37	0	0	3	37
Finished	54	5	9	7	13	1	2	11	20
Samples meeting standards§	33	4	12	5	15	1	3	8	24

*Samples collected during dry season only

†Number of samples positive for both enteroviruses and rotaviruses

‡NP—number positive

§Number of finished water samples that had a free chlorine residual and met USEPA turbidity and bacterial standards but were positive for viruses

TABLE 3
Identification of enterovirus isolate

Water Sample	Collection Date	Number of Isolates	Identification
Raw	3/82	5	Coxsackie B-5
		2	Polio 1
		2	Echo 24
		4	Echo 9
Clarified	3/82	3	Echo 9
		1	Polio 1
Finished	3/82	4	Coxsackie B-6
		4	Coxsackie B-5
		2	Polio 1

coli Hfr* as the host bacterium. Ten millilitres of each sample were assayed by the plaque technique using a soft-agar overlay method.¹⁷ Penicillin G† (300 mg/L) was added to samples containing high concentrations of bacteria before assay.

Quality assurance. Stool specimens or fecal swabs were collected from the laboratory personnel coming in contact with the samples during collection. All personnel wore disposable gloves when handling filters or samples throughout the course of analysis. The specimens were processed and inoculated onto cell cultures in designated areas physically separated from areas where laboratory strains of viruses were in use.

Results and discussion

The biological and physio-chemical characteristics of the water after the treatment steps were examined over a two-year period. Four sampling periods were chosen, three during the dry season (March 1982, January 1983, and April 1983) when the plant was operating optimally, and one during the rainy season (July 1982) when the plant could not produce water that met minimum coliform and turbidity standards.¹⁸

The rainy season is associated with a dramatic drop in raw water quality because of large amounts of sediment produced by stormwater runoff. This was evident by relatively high turbidity, bacteria, and coliphage counts in the raw water as well as in the finished water. Enterovirus isolation was not enhanced during the rainy season, perhaps because of lower concentration efficiency of the filters for enteroviruses caused by the high turbidity. One of the most dramatic changes was in rotavirus concentration in the finished water, which averaged 264 immunofluorescent foci (i.f.)/100 L, with 100 percent of the samples positive during the rainy season; only 13 percent of the finished water samples were positive during the dry season, averaging 2 i.f./100 L. The contrast in water quality produced during the dry and rainy seasons has been documented,¹⁹ thus further discussion is restricted to the three collections conducted during the dry season.

Nineteen samples were collected from raw water at the intake, 14 samples after prechlorination-clarification, 8 samples after filtration, and 54 samples from finished water after chlorination. Water was prechlorinated as it entered the plant, giving an average of 0.8 and 0.6 mg free chlorine/L in the water postclarification and postfiltration, respectively. After final chlorination, the finished water contained an average of 1.6 mg free chlorine/L, with only two samples having less than 0.5 mg/L.

The mean values for turbidity, bacteria, and viruses were calculated for

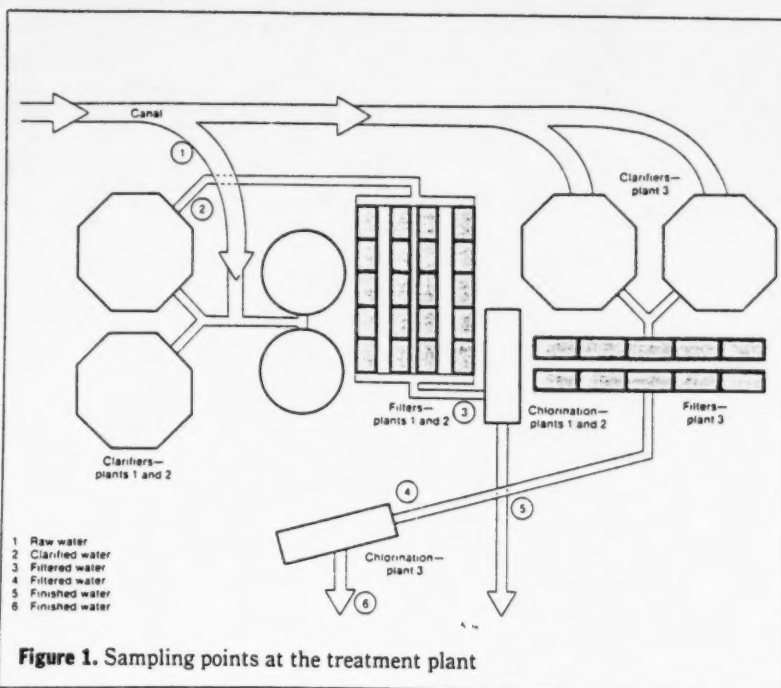


Figure 1. Sampling points at the treatment plant

each sampling trip (March 1982 and January and April 1983), as well as the combined means for the data from the raw, clarified, filtered, and finished water samples (Table 1). All means were weighted by the number of samples per trip. In general, a comparison of grand means showed that the total plate count bacteria were the most numerous, ranging from 9/mL in the finished water to 465/mL in the raw water, followed by the fecal streptococci (20–4452/100 mL), enterococci (6–1165/100 mL), total coliforms (1.5–216/100 mL), and fecal coliforms (1–66/100 mL).

It has been suggested that bacterial viruses, particularly coliphages, can be used as indicators for viral pollution. Coliphage concentrations, as determined from 1-L grab samples taken at each site, ranged from 1 to 1769/L in the finished and raw water, respectively, and correlated with all the bacterial numbers. The numbers found in the filter eluates, however, never equaled the numbers found in the direct sample. In some instances coliphages were detected in the direct grab sample but were not found in the concentrate. Several factors, such as inactivation during concentration, elution with the high-pH beef extract,^{19,20} or storage during transport to the laboratory, may have contributed to the absence of bacteriophage in the concentrate. Perhaps a modified procedure designed specifically for the concentration of bacteriophage may be more conducive to their detection in large volumes. With a more sensitive test, coliphages may be more useful for quantifying the viral pollution in waters.

Enteric viruses were isolated from all sites within the plant on all four collection trips. Comparison of samples collected during the dry season showed, however, that a high percentage of the positive samples for both enterovirus and rotavirus occurred during the March 1982 trip. Because it has been shown that viral isolates from sewage give an indication of the viruses present in the community,²¹ it may be inferred that increased levels of these viruses were present in the population at that time.

Table 2 summarizes the data on enteric virus isolation from all the samples. Viruses were recovered from 68 percent of the raw water samples; 50 percent and 37 percent of the samples were positive for viruses after clarification and filtration, respectively. Viruses were detected in 20 percent of the finished water samples. Even more significant, eight (24 percent) of the finished water samples that contained free chlorine levels >0.2 mg/L and that met turbidity and bacterial standards were also positive for enteric viruses. Only a small percentage of samples contained both enteroviruses and rotaviruses. Although an equal number of raw water samples contained each virus type, rotaviruses were more commonly isolated in the clarified, filtered, and finished waters.

The high fecal streptococcus levels in the raw water (giving a high ratio of fecal streptococci to fecal coliforms) indicate that much of the microbial contamination may have been of animal

*15597, American Type Culture Collection, Rockville, Md.
†KC Biological Inc., Lenexa, Kan.

TABLE 4
Average percent reductions of turbidity, bacteria, and viruses after successive drinking water treatments

Type of Water	Date	Turbidity ntu	Total Plate Count	Bacteria				Coliphage (Direct)	Coliphage (Concentrate)	Enterovirus CPE	Rotavirus
				Total Coliforms	Fecal Coliforms	Fecal Strep	Entero- cocci				
Prechlorinated-clarified	3/82	11	47.5	100.0	99.5	99.5	*	28.5	99.0		
	1/83	0†	89.1	94.3	100	92.6	97.3	97.0	99.91	25 78	95 0
Grand mean		4	78.9	98.9	97.1	97.2	97.3	90.4	99.7	55	61
Filtered	1/83	73	98.9	98.5	100	99.7	99.8	99.8	99.9	100	0
Finished	3/82	81	91.7	100	100	99.7	*	100	99.8	45	99
	1/83	76	98.6	98.6	100	99.5	99.5	99.9	99.9	92	0
	4/83	90	98.6	98.9	99.3	99.7	*	99.9	99.8	100	93
Grand mean		87	98.1	99.3	98.5	99.5	99.5	99.9	99.9	81	93

*Not determined

†No removal

‡Weighted by number of samples per trip

origin.¹⁵ Many animals excrete rotaviruses,²² thus it is possible that the rotaviruses detected were of animal origin. In a previous study at the same water treatment plant, however, neutralization tests performed on the rotavirus isolates indicated that most were of human origin.⁶

Enteroviruses were isolated from 18 samples and identified on BGM cells using the Lim-Melnick serum antibody pools. Of the 52 isolates, 9 were Coxsackie virus B5, 7 were echovirus 9, 5 were poliovirus 1, 4 were Coxsackie virus B6, and 2 were echovirus 24 (Table 3). Six isolates produced plaques but were not neutralized using the antibody pools.

All isolates initially produced CPE on a BGM monolayer and were then confirmed by production of CPE by a second passage on BGM cells and plaquing, using an agar overlay method. Eight of the isolates did not produce plaques but did yield CPE by a third passage on BGM cells. The 19 isolates that failed to produce plaques were examined using transmission electron microscopy (TEM). Volumes of approximately 0.1 mL from each sample (second passage) were fixed in paraformaldehyde and were negatively stained using phosphotungstic acid and viewed under a transmission electron microscope for virus particles. Eleven isolates were positive when examined by TEM and ranged in size from 22 to 30nm; further identification was not attempted. Two isolates were negative, perhaps because of an insufficient titer.

Average percent reductions for all water quality parameters studied were determined after each step in the treatment processes (Table 4). The major reduction in bacteria and coliphage (90-99 percent) came after prechlorination-clarification, with slight decreases during the other treatment steps. The initial reduction of microorganisms during the treatment process may be due to

the addition of chlorine, although adsorption to the floc during clarification is undoubtedly capable of removing large numbers of organisms. The largest reduction in turbidity occurred during filtration and averaged 87 percent after complete treatment.

The largest removal of enteroviruses appeared to occur during the prechlorination-clarification and filtration steps. The largest reduction in rotaviruses occurred after prechlorination-clarification and postchlorination of the finished water. Of all the groups of microorganisms studied, enteric viruses were the least effectively removed.

Although the plant was able to produce finished water with a turbidity of 1 ntu and <1 total coliform/100 mL, sanitary engineering evaluations showed major operational and design deficiencies. The most serious problem was in the filters, in which cracking, mudballs, and sandboils were observed, indicating short-circuiting. The plant may have been operating near or above its design capacity (hydraulically overloaded), but the extent of the problem could not be determined due to the lack of metering. In addition, according to the operators, during peak flow periods chlorine contact times were probably less than 30 min.

Recently, other investigators have also examined water treatment processes for their efficiency in removing enteroviruses.^{5,23} Payment and Trudel⁵ detected enteroviruses in 7 percent of the finished water samples collected at 7 drinking water treatment plants with an average removal rate of 99.98 percent. These authors also observed that no significant inactivation of enteroviruses was apparent after ozonation or final chlorination. In a study conducted by Stetler et al,²³ viruses were reduced 61 percent and 90 percent after clarification and filtration, respectively. No naturally occurring viruses were isolated in the finished

water, although the average free chlorine concentration in the finished water was greater than that reported in this study. The studies by Payment and Trudel⁵ and Stetler et al²³ demonstrated greater removals of turbidity, bacteria, and enteroviruses than this study, yet it was still apparent that the viruses were least efficiently removed at each stage of the treatment process.

No previous studies on the occurrence of rotaviruses in drinking water have been conducted. Because outbreaks of waterborne disease caused by rotaviruses have been documented,²⁴⁻²⁸ additional studies are needed to assess their occurrence in raw and treated drinking waters. In this study, the results suggest that rotaviruses may not be removed by filtration as efficiently as enteroviruses. In the laboratory, previous studies by Farrah⁷ and Steinmann and Havemeister²⁹ showed that the removal of Simian rotavirus SA-11 was noticeably less than the removal of poliovirus type 1 by aluminum hydroxide flocs. Farrah et al⁷ also reported that human rotavirus particles present in dilute stool suspensions were noticeably removed from water by alum flocs. Thus, both laboratory and field studies suggest that rotaviruses are less susceptible to removal by flocculation than enteroviruses. Although chlorine disinfection appears to be the most highly efficient barrier for rotaviruses, human rotaviruses have been shown to be the least sensitive to inactivation by chlorine, chlorine dioxide, and ozone, compared with some of the enteroviruses.³⁰

This study and others,^{2,3,5} suggest that enteric viruses can occur at detectable levels in finished drinking water that meets current coliform and turbidity standards and that contains >0.2 mg free chlorine/L. The isolation of human enteric viruses in these waters is not a condemnation of conventional treatment

practices but is an indication that water quality parameters currently accepted to ensure the production of microbially-safe water do not necessarily ensure the absence of enteric viruses.

Deficiencies in the 205-mgd (776-ML/d) treatment plant examined in this study could explain the occurrence of enteric viruses in the finished water. Still, it is significant that these deficiencies were not always reflected by the quality of the finished water during the dry season. It may be that, in marginally operated (i.e., those with operational deficiencies that may still meet standards) water treatment plants or in plants using a heavily contaminated raw water source, measurable levels of enteric viruses are able to penetrate the barriers afforded by the treatment process, even when current water quality standards are achieved.

It is difficult to define those operational conditions at a treatment plant under which all measurable levels of viruses would be removed, and to determine whether other microbial indicators such as coliphages would better reflect treatment removal of enteric viruses. This is because of the low isolation rate of enteric viruses in treated drinking water, which makes it difficult to establish sound statistical relationships. Further research should be conducted to define treatment conditions under which viruses may occur in finished drinking water, even though other water quality characteristics are satisfactory. Also, better indicators of treatment plant efficiency for enteric virus removal should be sought.

Conclusions

- Both enteroviruses and rotaviruses could be isolated from finished drinking water containing >0.2 mg/L levels of chlorine and meeting coliform bacteria (1/100 mL) and turbidity (1 ntu) standards.

- Enterovirus and rotavirus removal averaged 81 and 93 percent, respectively, for the complete treatment process involving prechlorination-flocculation, sand filtration, and chlorination.

- The complete water treatment process was more effective at removing turbidity, coliforms, fecal coliforms, fecal streptococci, standard plate count bacteria, and coliphages than at removing enteric viruses.

- Pilot-plant studies as well as field studies should be conducted to determine the occurrence and significance of enteric viruses in finished drinking water and to evaluate coliphages as an indicator system for animal viruses as well as other water quality parameters.

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